**ABSTRACT**

A crucial event in animal development is the specification of primordial germ cells (PGCs), which become the stem cells that create sperm and eggs. How PGCs are created provides a valuable paradigm for understanding stem cells in general. We find that the PGCs of the sea urchin *Strongylocentrotus purpuratus* exhibit broad transcriptional repression, yet enrichment for a set of inherited mRNAs. Enrichment of several germline determinants in the PGCs requires the RNA-binding protein Nanos to target the transcript that encodes CNOT6, a deadenylase, for degradation in the PGCs, thereby creating a stable environment for RNA. Misexpression of CNOT6 in the PGCs results in their failure to retain Seawi transcripts and Vasa protein. Conversely, broad knockdown of CNOT6 expands the domain of Seawi RNA as well as exogenous reporters. Thus, Nanos-dependent spatially restricted CNOT6 differential expression is used to selectively localize germline RNAs to the PGCs. Our findings support a ‘time capsule’ model of germline determination, whereby the PGCs are insulated from differentiation by retaining the molecular characteristics of the totipotent egg and early embryo.

**KEY WORDS:** Germ line, PGC, CNOT6, CCR4, Nanos, Pumilio, Transcriptomics, Sea urchin

**INTRODUCTION**

The germ line provides an immortal link between generations by transmitting heritable information from parent to progeny. Specification of the animal germ line typically occurs during embryogenesis, when primordial germ cells (PGCs) fated to become the gamete-producing stem cells of the adult are segregated from somatic lineages. PGCs in the numerous species studied share common molecular signatures, including the RNA helicase Vasa, the translational repressor Nanos and the Argonaute family member Piwi (Ewen-Campen et al., 2010). Surprisingly, despite this conservation of gene expression, the developmental routes that lead to it are remarkably diverse. Strategies for PGC segregation can be considered within a continuum of inherited and inductive mechanisms. An example of the inherited mode is that of *Drosophila melanogaster*, the PGCs of which are the first cells to form in the embryo. Their specification involves maternally supplied determinant mRNAs and proteins, collectively called germ plasm, which is actively transported and inherited by the presumptive PGCs. Conversely, PGCs in the mouse are specified by inductive signaling originating from the extraembryonic ectoderm. Most knowledge regarding these disparate mechanisms comes from studies in *Drosophila*, *Caenorhabditis elegans*, zebrafish and mice (Ewen-Campen et al., 2010; Pehrson and Cohen, 1986; Seydoux and Braun, 2006; Tanaka and Dan, 1990; Yajima and Wessel, 2011, 2012).

Comparatively little is known about PGCs outside these groups. Echinoderms, a large and diverse phylum, form part of the sister group to the chordates. The best-studied examples of this group are the sea urchins, including *Strongylocentrotus purpuratus*. In this species, four PGCs called small micromeres (sMics) are created by an asymmetric division at the fifth embryonic cleavage (Ewen-Campen et al., 2010; Juliano et al., 2006; Pehrson and Cohen, 1986; Seydoux and Braun, 2006; Tanaka and Dan, 1990; Yajima and Wessel, 2011, 2012) (Fig. 3C). After their formation, the sMics divide once during gastrulation to give eight descendants. These cells then assort into larval niches called the coelomic pouches, which are the major contributors to the juvenile sea urchin (Pehrson and Cohen, 1986; Tanaka and Dan, 1990). The early creation of the sMics is perhaps suggestive of inherited specification; however, sea urchins do not possess a classically defined germ plasm of aggregated germ line determinants. Instead, germline RNAs, including those of Vasa and Seawi (a Piwi family member), are maternally deposited, broadly distributed in early embryos and later refined to the sMics during gastrulation (Juliano et al., 2006; Seydoux and Braun, 2006).

To better understand the specification of the sMics, we used a transcriptomic approach to identify sMic-enriched mRNAs. We learned that the sMics are broadly transcriptionally repressed and identified transcripts, like Vasa and Seawi, that are ubiquitous in the early embryo but are later turned over in somatic cells to result in sMic enrichment. The expression dynamics of these discovered transcripts imply a post-transcriptional mechanism by which sMics retain RNA. Transcriptome analysis identified the mRNA encoding the CCR4-related deadenylase CNOT6 as uniquely depleted in the sMics. This depletion is dependent upon the RNA-binding protein Nanos and sequence elements in the CNOT6 3′ UTR that match the highly conserved binding consensus for Pumilio, the binding partner of Nanos. Depletion of CNOT6 is required for retention of Vasa protein and Seawi RNA in the sMics.

**RESULTS**

**Differential expression analysis and identification of sMic-enriched transcripts**

We developed a method for isolating PGCs *en masse*. In *S. purpuratus*, the sMics selectively retain the fluorescent dye calcein due to altered multidrug transporter activity (Campanale and Hamdoun, 2012) (Fig. 1A,B), even when dissociated into single-cell suspensions (Fig. 1C). We purified sMics by fluorescence-activated cell sorting (FACS), which yielded approximately 0.5%...
of the total population of the embryo (Fig. 1D; supplementary material Fig. S1A-F). By qPCR, isolated calcein-positive cells were 16-fold enriched for Nanos, a known sMic-specific transcript, but were not enriched for Spec, an ectodermal transcript (supplementary material Fig. S1G). With this indication of purity, total RNA was then isolated and deep-sequenced without amplification from three biological replicates: isolated sMics, non-sMics and disaggregated whole embryos. After assessing variation between samples by multidimensional scaling (MDS) analysis, we performed differential expression analysis to discover sMic-enriched and -depleted transcripts (supplementary material Fig. S1H-J). In summary, with a significance cutoff of 0.05 (false discovery rate, FDR), we identified a union set of 230 differentially expressed transcripts (both sMic enriched and depleted) between these comparisons (Fig. 1E; supplementary material Fig. S1I; Tables S7-S9).

The sMic-enriched transcripts included Nanos and Delta, which have previously been identified as sMic-localized, as well as SpG-cadherin, which is required for sMic fate in *S. purpuratus* and is enriched in the sMics of *Lytechinus varieatus* (Juliano et al., 2010; Miller and McClay, 1997; Oliveri et al., 2002; Yajima and Wessel, 2012). The sMic-enriched transcripts fell into diverse functional categories, but transcriptional regulation and RNA binding were overrepresented according to a gene set enrichment analysis (supplementary material Table S1). Several sMic transcripts lie within the same pathway. For example, we identified the DNA-binding factor Baf250 and the ATPase Brg1, which both assemble into the pluripotency-associated esBAF chromatin remodeling complex (Lessard and Crabtree, 2010). In addition to Delta, we also identified MibL, a potential regulator of Notch/Delta signaling (Le Borgne and Schweisguth, 2003).

![Fig. 1. FACS isolation and deep sequencing of sMics. (A) Schematic diagram depicting FACS isolation, RNA deep sequencing and differential expression analysis. (B) sMics accumulate the fluorescent dye calcein at 15 hpf. sMics are co-labeled with an mCherry fusion reporter of Vasa, a conserved germine RNA helicase that localizes post-translationally. (C) Calcein labeling is retained in blastomeres dissociated at 15 hpf. Arrows indicate labeled sMic. (D) Representative scatter plot of sMics collected by FACS, plotted as forward scatter versus calcein fluorescence. Cells collected as putative sMics are indicated by the box, which represents 0.5% of the total cell sample. (E) Venn diagram summarizing the number of sMic-enriched and -depleted transcripts discovered in three differential expression comparisons with FDR<0.05: sMic versus whole embryo (WE) and sMic versus non-sMics (NS), with and without replicate 1 included. Transcript totals are given as sMic-enriched/sMic-depleted. Scale bar: 20 µm.

The sMics are broadly transcriptionally repressed

Surprisingly, the majority of sMic-enriched transcripts we discovered appear to be maternally deposited. To examine temporal expression dynamics of sMic-enriched genes, we used a microarray dataset containing the whole-embryo expression level of all genes at multiple time points (Wei et al., 2006). On average, sMic transcripts were at maximum abundance at 2 and 15 hours post fertilization (hpf) (Fig. 2A). sMic transcripts then sharply dropped in abundance at 30 hpf, just after the onset of gastrulation. By calculating the 30 h/15 h abundance ratio, we found that sMic transcripts were statistically overrepresented for decreasing abundance compared with the whole-transcriptome average and with sMic-depleted transcripts (Fig. 2B). These dynamics suggest maternal loading followed by broad turnover. sMic nuclei are depleted for RNA polymerase II phosphorylated at serine 2 of the C-terminal domain, a marker of transcriptional elongation. sMic nuclei are highly enriched for histone 3 lysine 9 trimethylation (H3K9me3), a heterochromatin marker (Fig. 2E,F). Thus, we infer that sMics are transcriptionally repressed relative to their somatic neighbors. Furthermore, we did not detect transcripts that accumulated exclusively in the sMics. In this regard, Nanos remains the unique exception (Fig. 3A). Rather, sMic transcripts were broadly detectable in eggs and early embryos until blastula-to-gastrula stage, when broad turnover occurs in all cells except the sMics. Such genes include Baf250, Cdspl2/SCP2 [an RNA polymerase II (RNAPII) C-terminal domain phosphatase], z62 (a C2H2 zinc-finger protein) and MibL (Fig. 3B; supplementary material Fig. S2A-F). These observations suggest that sMics retain maternally loaded and zygotically transcribed transcripts, which are cleared from somatic cells during the blastula-to-gastrula transition. Given this repression...
and the sMic transcript dynamics, we suggest that sMics inherit and retain their select mRNAs rather than actively transcribing them (with the important exception of Nanos).

**CNOT6 transcript is selectively degraded in the sMics by a Nanos/Pumilio-dependent mechanism**

Our transcriptome analysis and in situ hybridizations imply a mechanism by which the sMics stably retain inherited RNA. In addition, we previously found that RNA microinjected into the fertilized egg is degraded in resultant somatic cells but retained in sMics, independently of its sequence (Gustafson and Wessel, 2010; Oulhen and Wessel, 2013). Our study offers an explanation for these phenomena: the top sMic-depleted transcript encodes CNOT6 (FDR=2.40E-13), an ortholog of CCR4 and a broadly conserved deadenylase subunit of the conserved CCR4/POP2/NOT complex (Collart and Panasenko, 2012). As a major regulator of RNA stability, depletion of CNOT6 could enhance RNA retention.

By fluorescence in situ hybridization (FISH), CNOT6 transcript is detectable ubiquitously in eggs through 32/60-cell embryos (Fig. 3D-F), but the transcript is uniquely depleted in the sMics by blastula stage (Fig. 3G-I). Nanos is a strong candidate for mediating this depletion. In diverse species, Nanos and its binding partner Pumilio recognize highly conserved motifs in the 3’ UTRs of target transcripts, termed Pumilio response elements (PREs), leading to mRNA degradation (Chen et al., 2012; Gerber et al., 2006; White et al., 2001; Wreden et al., 1997). The three S. purpuratus Nanos paralogs are each expressed in the sMics and are required for their survival (Juliano et al., 2010). Furthermore, we find that the 3’ UTR of CNOT6 contains two PRE sequences, suggesting that it is a Nanos/Pumilio target (supplementary material Fig. S3). We therefore knocked down Nanos with a translation-blocking morpholino antisense oligo (MASO) targeting the two most abundant of the three paralogs. This previously characterized MASO results in the eventual death of the sMics, and its effects were rescued by expression of a MASO-insensitive Nanos construct, thereby demonstrating specificity (Juliano et al., 2010).

In morphant embryos, CNOT6 mRNA accumulated in sMics (Fig. 4A,B). We next tested the two putative PREs as sequence-specific targets with MASOs complementary to these two sites, which we predicted would occlude binding of Nanos/Pumilio. Consistent with Nanos knockdown, PRE-protecting MASOs caused retention of CNOT6 mRNA in the sMics (Fig. 4C). To further test the motifs, we used reporter constructs containing either the full-length wild-type CNOT6 3’ UTR, or the full-length UTR with the PREs mutated singly or in combination (Fig. 4D-H). The wild-type reporter recapitulated the sMic exclusion of the endogenous transcript (Fig. 4E); however, mutations of the PREs resulted in sMic retention (Fig. 4F-H). In further support of its role, Pumilio co-immunoprecipitates with Nanos (supplementary material Fig. S4A). We attempted to test the effect of Pumilio knockdown on CNOT6 accumulation; however, these embryos were developmentally arrested before blastula stage, probably pointing to pleiotropic effects (data not shown). Indeed, Nanos-independent roles for Pumilio have been identified (Van Etten et al., 2012; Weidmann and Goldstrohm, 2012). Indicative of its diverse functions, Pumilio protein is detectable in granules in all cells of the sMics.
early blastula and is highly enriched in the Veg2 mesodermal precursors of later blastulae (supplementary material Fig. S4C,D). Nanos-mediated degradation of CNOT6 transcript is surprising, especially in light of evidence that Nanos functions by recruiting the CCR4-NOT complex itself (Suzuki et al., 2012). One possibility is that the CCR4-NOT complex maintains functionality with only the Pop2-related nuclease CNOT7, which is present in sMics at the transcript level (supplementary material Fig. S2G). Alternatively, maternal CNOT6 protein may be initially available in the sMics to degrade the mRNA, but then turned over later. Both possibilities are consistent with our conclusion that Nanos/Pumilio directs degradation of maternal CNOT6 mRNA in the sMics via PRE motifs in its 3′ UTR.

CNOT6 repression is required for retention of germline determinants

To test the requirement of CNOT6 repression for germline fate, we misexpressed CNOT6 in the sMics by multiple approaches. A CNOT6::mCherry fusion construct that expresses in all cells significantly reduced accumulation of a previously characterized sMic reporter, Vasa::GFP (Fig. 5A-C) (Gustafson et al., 2011). We next tested the accumulation of endogenous Vasa protein. Embryos were fixed at 42 hpf, following gastrulation and one division of the sMics, resulting in approximately eight descendants on average. Both CNOT6::mCherry, as well as PRE-protecting MASOs, predicted to stabilize endogenous CNOT6 in the sMics, resulted in significantly fewer Vasa protein-positive sMics (Fig. 5D-G). This observation could be explained by a failure in mitosis, cell death or loss of Vasa protein in the sMics. To distinguish between these possibilities, we stably labeled the sMic lineage by 5-ethynyl-2′-deoxyuridine (EdU) incorporation. Due to their slow cell cycle, sMics retain EdU pulsed before first cleavage, compared with other more rapidly dividing cells, enabling definitive lineage analysis (Tanaka and Dan, 1990). Both control and CNOT6-overexpressing embryos possessed similar numbers of sMics, indicating a loss of inherited determinants, but no other defects (Fig. 6D). Endogenous Vasa is probably lost later than the reporter construct (Fig. 5A-C) because of the abundance of maternally supplied Vasa protein, whereas injection of reporter RNA requires new translation (Voronina et al., 2008). Additionally, we tested the transcript abundance of the endogenous Argonaute family member Seawi in the sMics with CNOT6 overexpression. Seawi transcript is normally present in all cells but is highly enriched in the sMics (Yajima et al., 2013). With CNOT6::mCherry expression, the sMics lose enrichment for Seawi RNA (Fig. 6A-C). We conclude that CNOT6 depletion is necessary for retention of germline determinants in the sMics.

Repression of the CNOT6 deadenylase may allow for increased background stability for inherited RNAs in the sMics. Therefore, we predicted that global CNOT6 knockdown would expand the domain of RNA retention. CNOT6 is required for normal development of the embryo; strong knockdown leads to profound endomesodermal defects (supplementary material Fig. S4E,F). We therefore tested
the effects of weaker CNOT6 knockdown (under which development proceeds relatively normally) on Seawi transcript localization. In control embryos, Seawi transcripts are highly enriched in the sMics. However, when we globally reduce CNOT6 protein with either of two non-overlapping MASOs, Seawi transcripts are more broadly retained throughout the endomesoderm and oral ectoderm (Fig. 6E-H). To test the generality of CNOT6-mediated RNA retention, we used exogenous RNA encoding mCherry with an SV40 3′ polyadenylation signal. As reported for other exogenous RNAs, this transcript is retained in the sMics but is degraded in somatic cells in a sequence-independent manner (Fig. 7A) (Gustafson and Wessel, 2010; Oulhen and Wessel, 2013). However, when CNOT6 is globally depleted, mCherry RNA is retained throughout the endomesoderm (Fig. 7B,C). Our results indicate that differential CNOT6 expression is crucial for proper accumulation of transcripts within the sMics and is required for normal development of somatic lineages.

**DISCUSSION**

Our study reveals mechanistic insight into the divergence of germ line from soma. Uniformly dispersed mRNAs in the early embryo become highly asymmetric by the selective Nanos/Pumilio-mediated repression of CNOT6 in the germ cell precursors. This paradigm explains the localization of known sMic-enriched mRNAs, including Vasa and Seawi (Juliano et al., 2006), as well as foreign transcripts introduced in the early embryo (Gustafson and Wessel, 2010; Oulhen and Wessel, 2013). The RNA retention mechanism via CNOT6 depletion raises the question: is there specificity in the RNAs that sMics retain or is the retention completely nonselective? Although our dataset is probably incomplete, if retention were completely nonselective, one would expect to identify more than the 78 sMic-enriched transcripts we report. It is possible that the sMics possess mechanisms to exclude RNAs – Nanos/Pumilio is one such example, although there might be others that remain uncharacterized. Indeed, through bioinformatics we identified numerous transcripts that are depleted in the sMics, and it will be important in the future to investigate the mechanisms of their depletion. The fact that sMics generally retain RNA is probably necessary because they are transcriptionally quiescent. Transcriptional repression has also been documented in *Drosophila, C. elegans*, ascidians and mice, indicating that it is a fundamental feature of germline segregation (Nakamura and Seydoux, 2008; Shirae-Kurabayashi et al., 2011). Surprisingly, however, each organism achieves repression via distinct mechanisms. Although the precise nature of sMic repression is unknown, we find that the RNAPII phosphatase Ctdsp12 is sMic-enriched, indicating one possible mechanism.

Broad clearance of maternal RNA is a hallmark of the maternal to zygotic transition (MZT), a conserved event when developmental control is passed to the embryo. In *Drosophila*, there are two phases of degradation: the first occurs following egg activation and involves the RNA-binding protein Smaug, which recruits the CCR4-NOT complex to degrade diverse targets (Semetok et al., 2005). The PGCs possess degradation activity, but certain transcripts are protected in
the PGCs by motifs in their 3′ UTRs, perhaps by Oskar association (Bashirullah et al., 1999; Zaessinger et al., 2006). A second degradation process is driven by the mir-309 cluster at about 2 hpf (Bushati et al., 2008). The piRNA pathway also contributes to degradation of Nanos transcript in somatic cells and involves the CCR4-NOT complex (Rouget et al., 2010). In zebrafish, a primary effector of maternal RNA degradation at the MZT is mir-430 (Giraldez et al., 2006). It has been further observed that some mir-430 targets, such as Nanos, are degraded in the soma but protected in the PGCs (Mishima et al., 2006). In the sea urchin, the zygotic genome activates shortly after fertilization. However, the degradation aspect of the MZT in the future soma might be conserved via CNOT6 and could include small RNA mechanisms (Song et al., 2012).

Prior to this study, the only known mechanisms for stabilizing RNA in the germ line were via Dead end 1 (Dnd1) and Deleted in azoospermia-like (Dazl), which work by occluding microRNA-binding sites and by promoting deadenylation, respectively (Kedde et al., 2007; Mishima et al., 2006; Takeda et al., 2009). However, Dnd1 is not conserved outside of vertebrates. We show here in an early branching deuterostome that the deeply conserved deadenylase CNOT6 is repressed in its PGCs by Nanos/Pumilio, allowing for stable retention of inherited transcripts. As the PGCs are transcriptionally repressed, their inheritance might represent a ‘time capsule’ of early development; that is, they must subsist solely on the mRNAs they retain from the egg, independently of a germ plasm (supplementary material Fig. S5). A consequence of this strategy might be that the PGCs remain insulated from differentiation into somatic lineages. Furthermore, our model is consistent with an immortal cytoplasm hypothesis for the evolutionary origin of the segregated germ line at the transition from unicellular to multicellular animal life. The ancestral single-celled organism probably possessed the highly conserved factors found in animal germ lines, which were retained at the transition to multicellularity (Extaavour and Akam, 2003). It is the somatic cells that acquired unique characters to diversify from the original, progenitor cell type, while sacrificing reproductive potential (Buss, 1987; Extaavour, 2007). The diversification of the soma may have necessitated the evolution of global turnover events (e.g. the MZT) to eliminate RNAs associated with the egg. Instead of acquiring gametogenic capability anew, the embryonic germ cells remain protected and retain the characteristics of the egg. Downregulation of deadenylase activity provides a mechanism for understanding how cytoplasm that confers gametogenic potential is preserved in the segregated germ line.

**MATERIALS AND METHODS**

**Animals**

*S. purpuratus* were maintained in aquaria containing artificial seawater at 16°C. Individuals were induced to shed gametes by shaking or by injection of 0.5 M KCl. Eggs were collected in filtered seawater (FSW) and sperm was collected dry. Eggs were fertilized in the presence of 1 mM 3-aminotriazol (3-AT) (Sigma) to prevent crosslinking of fertilization envelopes. Embryos were reared at 15°C at a density of about 0.2% (packed egg volume/seawater volume) in stirring culture vessels.

**FACS isolation of sMics**

Embryos were collected 15 hpf by straining through 45 µm Nitex and concentrated to about 0.5% density in 50 ml FSW. PSC833 (Novartis) and calcine AM (C-AM, Invitrogen) were added to the FSW at 500 nM and 250 nM, respectively. The embryos were then incubated for 90 min at 15°C with constant rotation in 50 ml conical tubes. Embryos were pelleted by centrifuging at 250 g for 30 s, washed twice in 50 ml of calcium-free seawater and then resuspended in a 10 ml 1 M glycine 25 mM EDTA solution. After incubating for 5 min on ice, the embryos were disaggregated by trituration through a transfer pipette 20 times. The single-cell suspension was pelleted...
by centrifugation at 250 g for 5 min at 4°C, washed three times in calcium-
free seawater to a final sample volume of 4 ml. PSC833 was then added to
1 μM final concentration. The cell suspension was sorted on a FACSAria
instrument (BD Biosciences) set to 4°C. For long sorts, staggered cultures
were fertilized at 2 h intervals, and then labeled and disaggregated at 15 hpf
to avoid cell death and changes to their transcriptional profile. Cells were first
gated by forward and side scatter to remove debris and aggregates, and then
sorted by fluorescence intensity versus forward scatter. Cells were sorted
directly into 0.75 ml Trizol LS (Invitrogen) until the total volume reached
1 ml. Sorts, numbers of collected cells and RNA extraction yields are
summarized in supplementary material Table S2.

**Helicos sample preparation and deep sequencing**

RNA was extracted using Trizol LS reagent as described by the manufacturer
(Invitrogen). RNA was treated using RQ1 DNAse (Promega) for 30 min at
37°C, then extracted with acid phenol:chloroform (Ambion). Three
biological replicates of paired sMic, non-sMic and whole-embryo RNA
were collected. Two replicate pairs were pooled from three separate sorts,
whereas the third was collected from a single sort. Total RNA was stored in
100% ethanol, processed for RNA-seq without amplification or poly-A
selection and sequenced by Helicos tSMS (Lipson et al., 2009). These
quantitative short Helicos reads (averaging 30-35 nucleotides) were then
mapped and counted to a de novo reference transcriptome generated using

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Fig. 6. CNOT6 mediates selective enrichment of Seawi transcript in the sMics. (A,B) Expression of CNOT6::mCherry in all cells reduces the enrichment of
Seawi transcript in the sMics, detected by FISH (green), compared with mCherry alone controls. smic lineage is traced by EdU incorporation (red). (C) Pixel
intensity quantitation of Seawi transcript enrichment in sMics relative to the endoderm. (D) Counts of EdU-positive cells in mCherry control and CNOT6::mCherry-
expressing embryos indicate no change in sMic numbers. sMics were counted by focusing through the gut tip: one representative focal plane is shown in B.
(E-G) CNOT6 knockdown with either of two non-overlapping MASOs expands the domain of Seawi RNA into the endoderm and oral ectoderm at 42 hpf. (H) Fold
change of Seawi FISH average pixel intensity in control and knockdown embryos, relative to sMic Seawi intensity. P-values are calculated by unpaired two-tailed t-test. Scale bars: 20 μm.

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Fig. 7. CNOT6 regulates general retention of exogenous RNA in the sMics. (A,B) FISH for injected RNA containing the mCherry open reading frame and SV40 3′ polyadenylation
signal. This transcript is normally retained only in sMics at 96 hpf (A), but is retained broadly throughout the endomesoderm with
CNOT6 knockdown (B). (C) qPCR for exogenous mCherry RNA at 96 hpf. With
CNOT6 knockdown, mCherry levels increase twofold. Scale bars: 20 μm.
Illumina technology (described below). Helicos run statistics are listed in supplementary material Table S3.

Illumina sample preparation, deep sequencing and reference transcriptome assembly
RNA was extracted from several developmental stages, including ovary, 32-cell stage, 15 h blastula, 41 h gastrula and 4-day pluteus, using the RNEasy Mini kit (Qiagen) with on-column DNase. The isolated RNA was processed through standard procedures using the Illumina mRNA-Seq kit and sequenced on a single lane of a GAIIx using a read length of 105 bp with paired ends. The transcriptome was assembled using Velvet (1.0.09) and Oases (0.1.14) with a k-mer of 31 (Schulz et al., 2012). Exemplar sequences were selected from each locus based on abundance with a minimum length cutoff. The exemplar sequences were annotated with Blast2GO and compared by BLAST (minimum score of 1E-5) with the S. purpuratus SPU gene predictions (Conesa et al., 2005; Sodergren et al., 2006). All Helicos reads, de novo transcriptome reads and the assembled transcriptome are available under BioProject PRJNA188114.

Whole-mount in situ hybridization (WMISH) and immunofluorescence
WMISH was performed as described previously (Juliano et al., 2006). NanoS probe generation is described by Juliano et al. (2010). Approximately 1 kb antisense probe templates were PCR-amplified from cDNA using a reverse primer tagged with the T7 promoter and the T7 promoter. Digoxigenin-labeled antisense probes were transcribed using the Roche DIG RNA labeling kit according to the manufacturer’s instructions. Embryos of mixed developmental stages were fixed with MOPS-buffered PFA and hybridized for at least 5 days at 50°C with 70% formamide and 0.5 ng/µl probe. Hybridization was then visualized using either NBT/BCIP chromogenic detection or tyramide fluorescence amplification (TSA plus system, PerkinElmer). Vasa immunofluorescence was performed as described previously (Voronina et al., 2008). Rabbit antibodies to RNAP pSer2 and H3K9me3 were obtained from Abcam (ab5095 and ab8898, respectively; both 1:500). As the Vasa antibody was also raised in rabbit, co-labeling (e.g. Fig. 2) was performed as follows. Embryos were incubated with Vasa antibody (1:250) overnight at room temperature in PBST (0.1% Triton X-100, pH 7.4). The embryos were washed four times and then incubated 3 h at room temperature with rhodamine-labeled goat anti-rabbit Fab fragments. This procedure was then repeated sequentially with the pSer2 or H3K9me3 antibodies with FITC-labeled Fab fragments. Primer sequences used to amplify probe templates are listed in supplementary material Table S4. H3K9me3 antibodies with FITC-labeled Fab fragments. Primer sequences were as follows: F, 5′-CCCCGTAATGCAGAAGAAGA-3′; R, 5′-TCTTGCCCTTGAGTAGGTCGTC-3′.

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Competing interests
The authors declare no competing financial interests.

Author contributions
G.M.W. and S.Z.S. conceived the project; S.Z.S., J.P.C. and A.H. developed FACS methods; S.Z.S. and N.O. performed experiments. A.M.R. performed bioinformatics analyses; T.R. and P.M.M. performed Helicos sequencing; and S.Z.S. and G.M.W. wrote the manuscript, which was edited by A.M.R., N.O., J.P.C. and A.H.

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Supplementary material
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